PROTEIN QUALITY CONTROL IN ANIMAL MODELS OF ALS

by
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Abstract

Efficient and effective protein quality control are crucial components of cellular homeostasis in the presence of misfolded and aggregated proteins. One method for identifying the mechanistic targets of such protein quality control is via appropriate mouse models. Through exogenous alterations via methods such as drug injection on genetically modified mice, various crucial components of the protein quality control system may be identified. Such modulation could be induced in an effort to increase proteasome efficiency in clearing protein aggregates and alleviate dangerous levels of protein cytotoxicity. Thus in this experiment, a drug targeting the protein quality control system was administered to $SOD1^{G93A}$ transgenic mice to observe the potential alleviation of ALS symptoms. Drug efficacy was gauged based off of a rotarod balance test, grip strength test, and weight measurements. The drug's efficacy in vivo was the main objective of this research project. Additional research is required to understand the full effects of the drug and any potential confounding mechanisms.

Acknowledgments

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With regards to materials I am greatly appreciative of Adooq Biolabs for their supplying of Tenovin-6 and Sigma Aldrich for their DMSO and Cyclodextrin. I would also like to thank Bioline for their MyTaq Extract-PCR Kit and Promega for their GoTaq Polymerase, both of which were instrumental in providing clear, efficient, and successful mouse genotyping. Special thanks to Bioseb for their Grip Test apparatus and Harvard Apparatus for their RotaRod apparatus, both of which facilitated the acquisition of the data necessary for completion of this Master's Thesis.

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Introduction

Neurodegenerative disorders, though occurring less in the general population compared to other maladies such as cancer, heart disease, and chronic obstructive pulmonary disease (COPD), nonetheless generate significant fear among the vulnerable and the afflicted alike (Kinsella 1992). The general cause for such distress stems from the degenerative nature of such disorders, which not only induce functional loss of motor operations, but also steadily dissolve memories and personality. The four major neurodegenerative disorders: Alzheimer's, Parkinson's, Huntington's, and Amyotrophic Lateral Sclerosis (Lou Gehrig's Disease/ALS) all result in motor and cognitive loss, and are all presently untreatable (Shany-Ur and Rankin 2011). Of these disorders, ALS has been clinically documented to progress the fastest, with time of visible onset to fatality usually ranging from three to five years (Glicksman 2011). It is also extremely frightening; due to progressive muscle paralysis, patients generally die of suffocation as their respiratory muscles steadily shut down. The only FDA approved drug, riluzole, only extends patient life by one to three months (Dall'Igna, Bobermin et al. 2013).

Further complicating the issue of treatment is that the mechanistic effects and benefits of riluzole are poorly understood. Most researchers and clinicians gravitate towards the claim that it acts in dual-function manner, both accelerating glutamate uptake and clearance at neural synapses and reducing levels of neurotransmitter release as well (Blasco, Mavel et al. 2014). It has also been documented to block Tetrodotoxin (TTX)-sensitive sodium channels and directly inhibit kainite and *N*-methyl -D aspartate (NMDA) receptors (Dennys, Armstrong et al. 2015). TTX is a neurotoxin which binds to voltage-gated sodium channels in neuron membranes, thus preventing sodium ion permeability and firing of action potentials (Bane, Lehane et al. 2014). NMDA receptors are ion channels which are activated via glutamate or glycine binding, thus facilitating cation passage such as

sodium, calcium, and potassium ions (Liu and Zhang 2000). Frustratingly, over thirty Phase II and III clinical trials, many containing drugs with similar functions to riluzole have all failed to display significant clinical benefits (Genc and Ozdinler 2014). Consequently, there has been a surge in effort to identify another mechanistic pathway for novel drugs to target. A common target and purported cause of ALS is the gain-of-function mutation in the superoxide dismutase (SOD1) gene and protein, which facilitates its aggregation in motor neurons (Deng, Shi et al. 2006). The normal function of SOD1 is to convert toxic and dangerous superoxide radicals into less harmful forms such as molecular oxygen and hydrogen peroxide (Moreira, Pereira et al. 2013). Numerous studies have indicated that such aggregation can have numerous deleterious effects ranging from mitochondrial vacuolization to intracellular cytotoxicity by way of clogged and dysfunctional proteasomes (Martins and English 2014). It comes as no surprise that countless studies have struggled to alleviate ALS phenotype via extermination of SOD1 aggregates. One possible mechanistic target is the crucial p53 "guardian of the genome" protein, which is responsible for a plethora of functions, ranging from cell growth maintenance to most importantly for ALS, protein quality control (Periz, Lu et al. 2015).

Given its prevalence and precedent in previous ALS research, the SOD^{G93A} mutation is one of the most widely and densely studied in the field (Teuling, van Dis et al. 2008). Although its occurrence in humans is much more infrequent compared to other mutations, researchers hope that mechanistic discoveries of the mutation may hold significance for other mutations as well. However, beyond the scope of the various SOD1 mutations ranging from A4V to G85R, numerous other focal points are being scrutinized, such as C9orf72 and TDP43 (Ajroud-Driss and Siddique 2015). Nevertheless, one of the best models still remains the transgenic SOD^{G93A} mouse, having been studied in tremendous detail by countless laboratories. It is currently a standard mouse model in the ALS animal research field, and will be the main method of study for this thesis project.

The transgenic SOD^{G93A} mouse generally possesses over 20 copies of the mutant G93A transgene throughout its genome, which confers its strong ALS-related phenotype (Ohgomori, Yamada et al. 2016). Regardless, the model is not perfect, with some researchers claiming that such strong transgene presence and upregulation could present a confounding variable in itself (Pfohl, Halicek et al. 2015). Others suggest that such high protein levels could flood and overload cells, thus causing ALS-like symptoms from severe protein excess or other unknown mechanisms not mechanistically similar to clinical ALS (Pfohl, Halicek et al. 2015). Still, the mouse has been an invaluable tool in the ALS field and has helped pave the way for a multitude of discoveries and advancements.

Despite the depth of knowledge gained from this mouse model, many questions still remain as to the mechanisms of ALS progression and their fundamental cause. A commonly held belief is that ALS is caused by a toxic gain-of-function generated by the abnormal aggregation of mutant SOD1 proteins (Deng, Shi et al. 2006). Complete knockout of the SOD1 gene has also been proven to not cause ALS-like symptoms. A plethora of evidence supports these theories (Lobsiger, Boillee et al. 2013). Some labs have shown that innate structural deformities in mutant SOD such as G93A facilitate protein aggregation (Wright, Antonyuk et al. 2013). Others mention the ensuing intracellular chaos that occurs following aggregate formation (Karch, Prudencio et al. 2009). Unfortunately, with the consequent concern of what the SOD1 aggregates do to dysregulate cellular function and by what means, significant debate has been generated. Research has shown that among other acts, aggregates can accumulate at the mitochondria, ultimately causing vacuolation of the mitochondria and the release of many cytotoxic enzymes (Higgins, Jung et al. 2003).

Researchers have scrambled over the years to target as many mechanistic pathways and checkpoints as possible to halt the progression of ALS in SOD^{G93A} mice. Such efforts have included riluzole-like glutamate de-excitatory treatments, calcium regulation treatments, and modulations to

SOD1 regulation and levels (Prell, Lautenschlager et al. 2013). Many methods have seen improvement, yet fail to succeed in human trials. Whether a better target or a drug cocktail is needed has yet to be determined, but regardless, the search continues for another mechanism to halt or prevent mutant SOD1-related ALS.

One new focus has been to remove attention from alleviating SOD1 aggregate-related damages and instead concentrate on the source of the toxicity: the protein quality control system. Studies have indicated that proteasome functionality in the presence of $SOD1^{G93a}$ aggregates is diminished, which could not only allow further protein aggregate formation, but also interrupt numerous other intracellular regulatory processes (Kitamura, Inada et al. 2014). As such, a logical step would be to target the overarching control mechanism of cellular defense, the p53 tumor-suppressor protein. Some research has shown that p53 levels may already be quite high in the motor neurons of ALS patients, the consequence of cells struggling to remove the progressive buildup of insoluble protein aggregates (Turnquist, Horikawa et al. 2016). It is possible that aggregates may clog proteasome pores, thus diminishing overall protein regulatory efficiency. The subsequent buildup of aggregate concentration could have substantial and progressive deleterious effects on the cell, urging p53 levels high enough to induce eventual apoptosis (Kitamura, Inada et al. 2014).

Of curiosity is what modulation of the p53 level might do to affect protein quality control levels. It seems that this may depend on the stage in which p53 levels are altered. Too late in ALS progression and excess p53 levels could simply tip the scale towards immediate apoptosis, thus worsening symptoms. If p53 is elevated early enough in the diseases progression however, it could possibly confer a protective effect, allowing relatively healthy motor neurons to resist ALS progression to a measurable degree. The purpose of this thesis project is to determine if early p53 level increases do indeed reduce ALS phenotype progression or onset and if so to what extent.

Modulation will be done via intraperitoneal drug injections of the drug Tenovin-6, which serves to increase p53 levels via deactivation of a p53 gene deacetylase. Tenovin-6 (Fig. 1B) is a small molecule activator of p53 transcriptional activity and a more effective water-soluble derivative of Tenovin-1 (Fig. 1A). The drug enacts its effect via inhibition of the Sirtuin 1, 2, and 3 protein deacetylases. The main focus, sirtuin 1 (SIRT1), is an NAD^+-dependent class III histone deacetylase, which deacetylates p53 at lysine 382 and modulates numerous other histone/non-histone proteins as well, thus reducing p53 transcription and functionality (Sasca, Hahnel et al. 2014). The goal of this mechanism (Fig. 3A) during activation is to ultimately reduce p53 levels, thus leading to cell survival. One potential method of stalling ALS symptoms may be to increase p53 levels via SIRT1 inhibition. A complicating factor however would be to determine the proper dose of SIRT1 inhibitor necessary to upregulate protein quality control without inducing apoptosis. Although SIRT1 modification via Tenovin-6 and like drugs has been researched in tumor cells, it has yet to be tried in ALS-expressing cells (Ueno, Endo et al. 2014). However, following transfection of HEK293T cells with $SOD1^{G85R}$ DNA, a dose-dependent reduction in mutant SOD aggregates was observed.

The primary goal of this experiment is to see whether controlled exogenous drug-mediated upregulation of p53 can improve the protein quality control pathway to the extent of reducing or delaying ALS-related phenotype. Such alterations are to indicated via multiple quantifiable data points including weight gain and degeneration over time, two weekly test performance trends, and variations in visual phenotype over time.

Figure 1

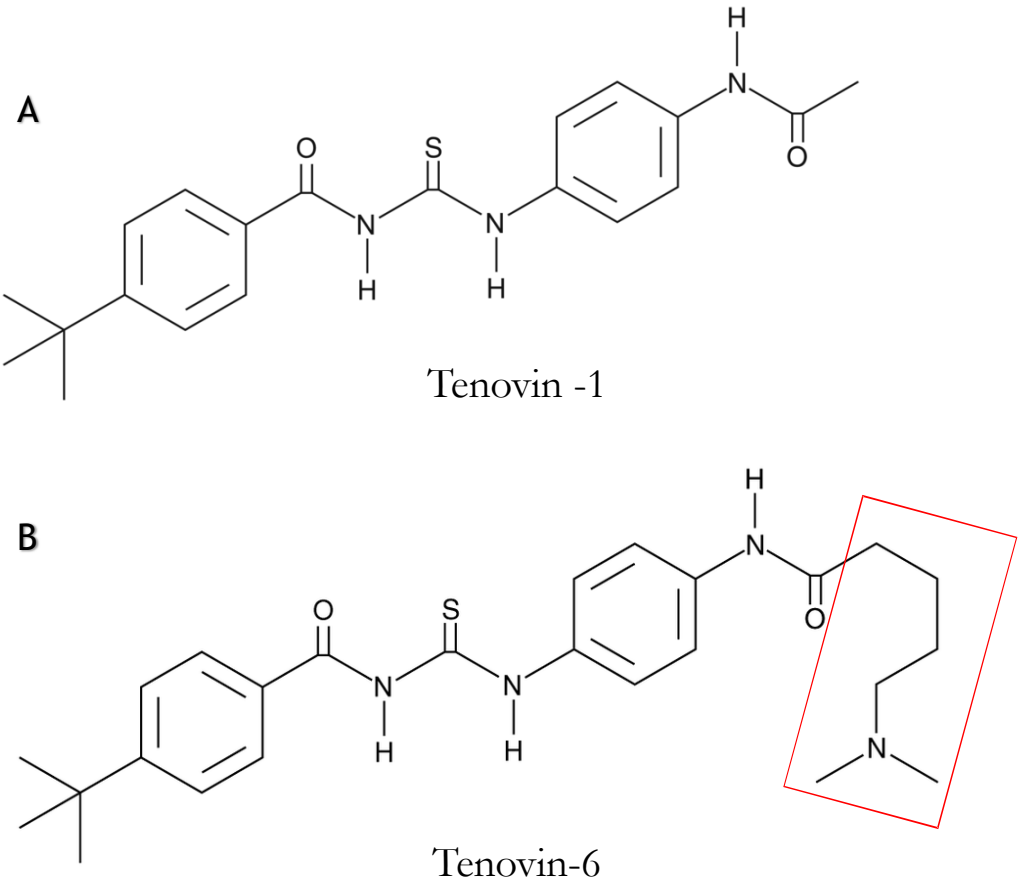

Figure 1: A*: The initial structure of Tenovin-1, a small molecular activator of p53. Although carrying potential beneficial effects in vitro, it is far too water-insoluble in vivo.*

B*: Tenovin-6 is synthesized via a modification of Tenovin-1 (highlighted in rectangle) which results in a 6 to-7-fold increase in water solubility in vivo, conferring clinical viability in mouse trials.*

(https://www.caymanchem.com/product/13086, 2016)

Figure 2

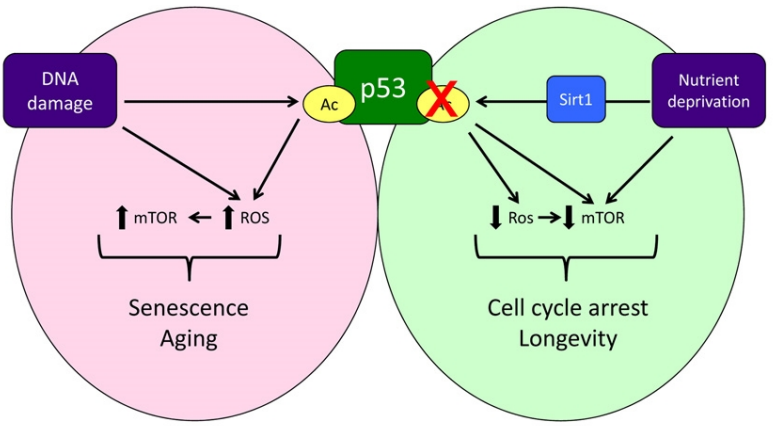

***Figure 2**: A general schematic of the sirtuin-1 pathway and its inhibitory effect via de-acetylation of p53. Tenovins 1 and 6 return p53 functionality via inhibition of sirtuin-1, although sirtuins 2 and 3 can be affected as well.*

(http://archive.impactaging.com/papers/v2/n8/full/100189.html, 2016)

Materials and Methods

Cell Culture

In order to verify the efficacy of Tenovin-6 in clearing mutant SOD1 aggregates, Human Embryonic Kidney 293T cells (HEK293T) were transfected with $SOD1^{G85R}$ DNA to instigate protein aggregate formation. All cells were maintained in a medium composed of: Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), β-Mercaptoethanol, non-essential amino acids (NEAA), and Glutamax.

Transfection

HEK293T cells were initially plated in six 60mm petri dishes, each pre-treated with 3mL of polyethylenimine (PEI) for thirty minutes. After the allotted incubation period, PEI and two subsequent washes with H2O were aspirated. The cell concentration for each plate was 4 mL of 1 x 10^5 cells/mL in the DMEM mixture. After a 24-hour incubation period, plates were rinsed and aspirated with 2.5mL of Opti-MEM (OMEM). A 0.5mL mixture of OMEM, lipofectamine2000, and BOS-$SOD1^{G85R}$ (1.54μg/μL) was then added to the six plates and incubated for five hours, upon which 2mL of DMEM/FBS was added per plate for a 24-hour incubation. At time of drug addition, six different drug conditions were created in separate 4mL batches. Plate 1 received DMSO alone as a control given that Tenovin-6 was diluted as stock in pure DMSO. Plates 2-5 received drug quantities allowing for plate concentrations in increments of 0.4μM up to 2μM. After another 24-hour incubation, all plates underwent a standard lysis protocol utilizing Buffer B, Iodoacetamide (IAA), and proteinase inhibitors. Lysate was then finally fractionated for use in western blot. Protein concentration was measured using a BCA protein concentration assay.

Western Blot

Western blots were run with pre-made 15% polyacrylamide gels each loaded with 40 μg of sample. Primary antibody mix was composed of 9mL of 5% BSA-TBST along with 3μL of SOD1-100 (1:3000) Rabbit and 3μL of β-actin. Secondary antibody was added for a 1:10000 dilution with anti-donkey rabbit 800 and anti-donkey mouse 680.

SOD^{G93A} Mouse Maintenance

$SOD1^{G93A}$ males were mated with WT Black-6 females for synthesis of gender/age/litter matched drug trial pairs and stock $SOD1^{G93A}$ positive (SOD+) males for mating purposes. All $SOD1^{G93A}$ negative (SOD-) and unmatched SOD+ females were discarded. A separate set of wild-type (WT) males and females were designated breeders for WT mating males and both WT and SOD mating females. Five matched WT pairs were also isolated for a drug vehicle control trial to serve as a testing and weight baseline. Except for underweight litters, all mice were split, weaned, tagged, tailed, and genotyped upon reaching 21 days of age. Upon genotyping and understanding of situational requirements, mice were sorted out appropriately.

DNA Extraction

Upon reaching 21 days of age, all SOD1 mice were sorted and tagged by gender and litter. Tails of 1mm in length were then cut and sampled for genotyping. For DNA extraction, a Bioline Mytaq Extraction Kit was utilized. Added to each sample were 10mL of Buffer A, 5mL of Buffer B, and 35mL of water. Subsequently, each tail-buffer mix was spun at 650rpm for 5 minutes at 75°C and then deactivated for 10 minutes at 95°C. The ensuing mix was then spun at 15000rpm in a centrifuge for one minute. Afterwards, 20μL of supernatant was transferred to a fresh 1.5mL

Eppendorf tube pre-filled with 180μL of Millipore H2O. This diluted DNA sample was then kept at -20°C for up to three months before being stored at -80°C indefinitely.

PCR

Upon complete DNA Extraction, samples were prepared for PCR using modifiable count 8-well strip individually capped PCR-tubes. A single reaction mix consisted of 2.25μL of extracted and diluted DNA, 3μL of MyTaq or GoTaq Polymerase, 0.25μL of WT primer (Forward and Reverse combined), and 0.5μL of G93A primer (Forward and Reverse combined). The PCR protocol itself was in accordance with Bioline.

DNA Agarose Gel

Upon PCR completion, a 1.5% agarose gel was molded with the appropriate amount of wells. Each sample run was accompanied with 1kb ladder along with a negative water control and a positive control if an initial run was unsatisfactory. Each well was loaded with the full 6μL PCR reaction with a running time of 12 minutes at 220V.

Intraperitoneal Injections

$SOD1^{G93A}$ mice were administered either drug or vehicle injections via intraperitoneal (IP) injections at 33±2 days every five days until ALS-induced fatality approximately 5-6 months later (18-20 injections total). Injection procedure and location were compliant with common research techniques and IACUC standards. No antiseptics were utilized prior to injection and no infections or early unforeseen deaths were observed. Injections were given every five days in contrast to weekly testing. This served a dual purpose; increased injection frequency coupled with variable days

of injection pre/post testing to eliminate any confound resulting from possible behavioral or molecular drug impacts.

Drug Mix

For low dose tenovin-6 injections of 80μM, 26.4μM, and 8μM, a drug cocktail was utilized in accordance with the set precedence of a previous cancer research paper. Each drug mix totaled 100μL (0.1cc) and consisted of: adequate drug quantity for the given dosage, DPBS, 10% DMSO, and 20% cyclodextrin. Drug mixes were made the day of injection with DPBS and Cyclodextrin stored at 4°C and DMSO and Cyclodextrin stored at -20°C. Insulin needles of 1cc capacity (26 gauge) were filled and cooled over dry ice until the moment of injection. Vehicle control injections replaced the removed drug stock with additional DPBS.

For the new Tenovin-6 4.5mM trial, a new and simpler protocol was instead adopted utilizing necessary drug amounts diluted in DPBS and 10% DMSO only. Cyclodextrin had been dropped from the mix given the favorable water-solubility of both drugs. For further efficiency and simplicity, pre-mixed injection stocks were formulated and stored at -20°C in aliquots of 1mL for future injection needle loading. Upon each thawing, 10 needles of 0.1cc each were loaded and returned to -20°C for easy daily acquisition. Like the previous protocol, needles were kept over dry ice prior to injection.

RotaRod Test Measurement

RotaRod testing was conducted weekly simultaneously with weight measurements. A quick single round training session was conducted the day prior to testing until proper acclimation to the machine was documented. Protocol was in similar adherence to that supplied by Biotrofix which composed of an initial 1-minute fixed-speed (12 rpm) run followed by a three-trial acceleration test

every hour. For texting, the machine was calibrated to begin at a speed of 4 rpm with a five-minute acceleration time to a top speed of 12 rpm; protocol was also in accordance with a previous study by Bennett et. Al. apart from a speed cap of 12 rpm as opposed to 40 rpm (Bennett, Mead et al. 2014). As with all testing, each mouse underwent three separate trials measured in seconds from start to fall, which were averaged out at the end of each testing session. Unfortunately, early weeks of testing generally show greater variance than in later rounds as younger mice are often inclined to jump and fall erratically. Such behaviors were eradicated via early training procedures.

Grip Test Measurement

Grip strength testing was enacted via a Bioseb grip strength apparatus. Similar to RotaRod testing, measurements were taken weekly, with each trial run averaging three separately recorded measurements. One potential measurement variation of note was the ability of mice to not only grab the apparatus, but also exert an opposing pulling force as well. Such actions often registered as an above expectation force, yet were considered to indicate their muscular proficiency. Measurements were taken on the scale of 25N, and like other recording mediums, all mean scores were eventually plotted on a time-lapse graph per subject mouse.

Weighing

Mice were weighed utilizing a laboratory grade portably electronic balance, which for the later 4.5mM Tenovin-6 trial was replaced with another balance without ill effect or deviation. Weekly weight measurements were taken to the hundredth of a gram and were later compiled on a time-lapse graph. Measurements were taken on the same day and time that RotaRod testing was carried out.

Phenotype Measurement

Mouse ALS phenotype was measured using two distinct methods beyond that of testing results and trends. The first was a self-produced three-stage scale which determined ALS stage based on overall appearance and motor deficiencies. The scale also possessed two early preliminary checkpoints, where stage one indicated a general though subtle disturbance to behavior and posture. Stage two indicated slightly more noticeable motor alterations not seen in WT mice at that age. Stage three represented a proper and clearly visible change in motor functionality characterized by shaky movement and impeded motor coordination. At this stage testing scores began to decrease with weight following immediately or shortly afterwards. Stage four was identifiable by severe gait and immensely diminished motor skills, with mice often scoring minimally on testing days. Stage five represented end-stage ALS, with subjects displaying nearly complete testing failure and almost full muscle paralysis. Mice at this stage were humanely euthanized days before full motor shutdown and death from starvation and dehydration.

A second a more general phenotype measurement is the leg splay reflex test which can be seen in Figure 3. As ALS progresses, mice, when held upside down by the tail spread their legs outwards less and less as motor functionality worsens. Its 0-3 score scale matched accordingly with the first novel 1-5 scale phenotype measurement used. In this second scale, a 0 score (Fig. 4.0) represented normal functionality while a score 1 (Fig. 3.1) indicated a slightly decrease in leg splay ability. Score 2 (Fig. 3.2) marked a significant decrease in muscular integrity while a score of 3 (Fig. 3.3) was characterized by near complete splay ability of the legs.

Euthanasia

Mice were euthanized humanely in accordance with standard IACUC protocols. Upon reaching terminal stages of ALS at approximately 150-180 days, mice were sacrificed via CO2 chamber and cervical dislocation.

Figure 3

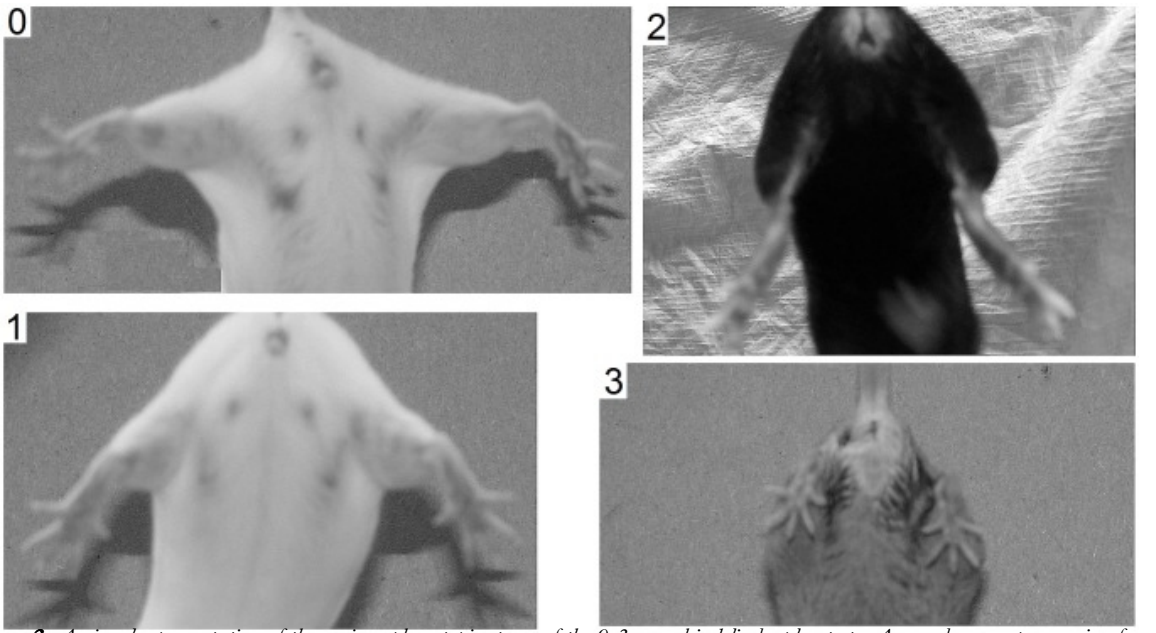

Figure 3: *A visual representation of the various phenotypic stages of the 0-3 score hind limb splay test. As can be seen, progression from 0 (healthy) to 3 (end-stage) displays a significant decrease in limb stability and integrity.*

0: Full limb functionality and muscular integrity
1: Slight degeneration in muscle and tensile strength
2: Severely diminished muscle function and splay ability
3: Fully lost splay ability

(https://www.researchgate.net/figure/263293067_fig1_The-splay-reflex-test-scored-on-a-0-3-scale-is-performed-by-lifting-the-mouse-by-its, 2016)

Tenovin-6 Low Dose SOD^{G93A} Transgenic Mouse Trial (n=30)

The low dose tenovin-6 mouse trial (n=30) was composed of three separate dose groups of 80μM, 26.4μM, and 8μM. Each group contained 5 pairs of mice, with each pair gender and litter matched with one mouse designated for drug injection and the other for vehicle injection only.

All three low dose Tenovin-6 dosages had no significant effect on age of death

Despite the variance in drug dosages across groups, there was no significant difference in the ages of end-stage death. Figures 4A-C all display slight differences in mean ages of death; however once all three groups were averaged together, mean ages of death became nearly identical between drug and vehicle groups despite dosage (Fig 4D). It is important to note that in accordance with IACUC humane treatment of animals, steps were taken to ensure mice did not die due to respiratory failure, starvation, or dehydration. Once mice reached stage 5 end-stage phenotype and were deemed fully immobile, they were sacrificed via CO2 and cervical dislocation.

All three low dose Tenovin-6 dosages had no significant effect on phenotype onset

In addition, experimental drug mice did not differ in onset for progression of visible ALS phenotype when compared to vehicle control mice on both measurement scales. The maximum stage 5 entry age difference between a pair of trial mice was approximately 10 days while the vast majority saw a difference of no more than three days. Regardless, any trends or differences proved insignificant; where a drug mouse in pair would reach stage 5 a week after the control, a drug mouse in another pair would reach stage 5 nearly a week prior to the control. Oftentimes when any discrepancies in phenotype checkpoint ages were seen, they were the result of noticeable weight differences (>2g) already present at birth prior to testing or injections. While no obvious or

significant differences were seen, neither were observed any unusual or unexpected phenotypes or behaviors. It was documented on random occasion however, that some mice would reach euthanasia requirements only days after reaching stage five, while others could endure for over two weeks. No trend was seen between pairs or groups and seemed to be unpredictable and was thus not pursued further.

Figure 4

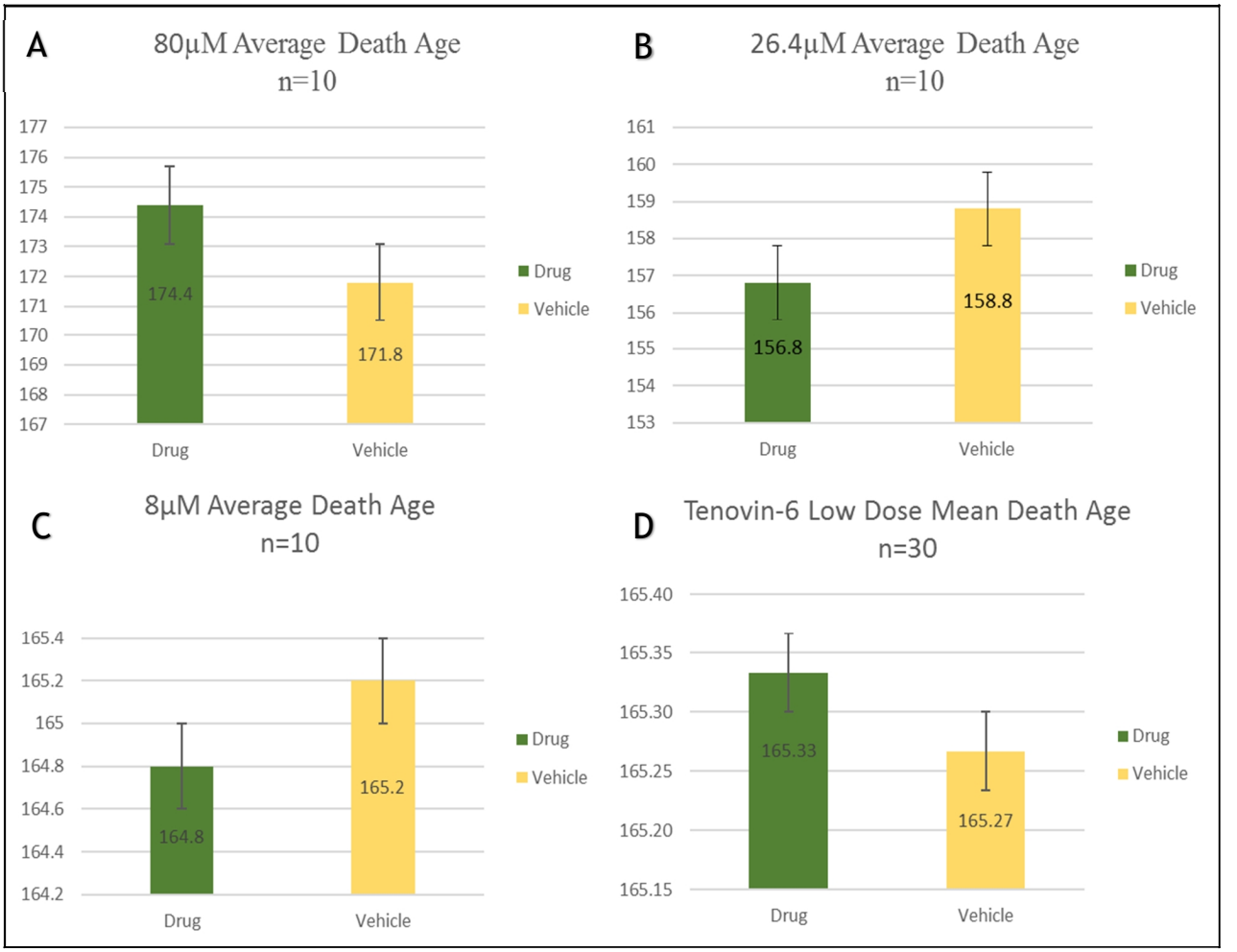

Figure 4: A+B+C*: All three drug groups (n=10 each) consisting of 5 drug/control pairs each were compared to one another based on age of death. While gender was mixed in groups, mice in each pair were both gender and litter matched. Analysis displayed a lack of trend and significance at p<.05 in death age.*

D*: A final analysis of all groups combined displayed almost identical mean age of death between both drug and control (vehicle) groups*

Tenovin-6 Baseline Control SOD1 WT Mouse Trial (n=10)

In addition to the three low dose tenovin-6 group, a fourth WT group was established as a potential baseline. Similar to the preceding drug groups, five properly matched mouse pairs were tested and monitored over the same timeframe as drug-group mice. Experimental mice received vehicle control injections while control mice received no injections or modulations to remain as unmodified as possible.

Comparing baseline WT and SOD1^{G93A} trials shows no trend disturbances prior to ALS onset

Given the concern that potentially even vehicle injections could cause unforeseen alterations to mouse physiology and genome, the baseline WT trial control group was studied, compared, and subjected to the same protocols as the low dose tenovin-6 experimental drug groups. Testing, weighing, and vehicle injections were terminated shortly after the death of the oldest drug trial mouse. All three tenovin-6 dosage groups exhibited the same weight trends within and between pairs, with either a pause or decrease in weight gain approximately beginning at week 10. Although the WT pairs do not display a pause or regression in weight gain due to absence of the G93A transgene, it is worth noting that there is no significant change in weight gain trends induced by injections.

Tenovin-6 Baseline Mutant Female SOD^{G93A} Transgenic Mouse Trial (n=5)

For further understanding of baseline SOD1 mutant mouse performance during weekly testing and weighing, five individual female SOD1^{G93A} positive mice were placed into a separate testing group. All were tested and weighed weekly, but were never injected or contrasted with any litter or gender

matched mice. This group was simply utilized as a quick comparison if in the unlikely event that vehicle injections alone served to skew testing/weight/onset data.

Non-injected SOD1^{G93A} trial females exhibit same trends as injected SOD1^{G93A} trial females

Analysis between the various trial groups displayed no significant differences between drug or vehicle control SOD1 positive mice and the non-injected SOD1 positive mice. Trends in weight gain, testing improvements and regression, and visual phenotype checkpoints remained similar among all groups.

Tenovin-6 High Dose SOD^{G93A} Transgenic Mouse Trial (n=10)

Given the indiscernible differences between pairs and groups of the previous low dose tenovin-6 trial, a new high dose trial was synthesized utilizing slightly different parameters. As mentioned prior, cyclodextrin was removed from injection mixes given the high water-solubility of Tenovin-6. Unfortunately, at the time of thesis presentation, this trial had not yet reached end stage. As such, both age of death and time of various late-stage phenotype onsets were unable to be quantified and compared. Instead, mid-stage visual phenotype onset was able to be recorded as well as testing and weight trends. An interesting find in the initial low dose tenovin-6 trial was that at approximately between the 10th and 12th weeks of testing, mice reached peak testing performance and weight and began to degenerate. This checkpoint was able to be observed before formulation of this thesis.

Tenovin-6 4.5mM drug mice exhibit early boost in RotaRod scorings

As evident in Figure 7, both drug and control groups displayed relative uniformity in RotaRod testing trends. Gender imparted no advantage or trial score extension. Interestingly however, the

experimental drug group saw a marked increase in performance on week 4 when contrasted with the vehicle controls. This score increase quickly normalized the following week, with testing trends up to week 10 remaining similar. Nevertheless, despite the week 4 score increase, the overall trends between the two groups did not prove to be significantly different. A proper follow-up would be to observe continued trends to end-stage phenotype and to mark trial performance peaks.

Tenovin-6 4.5mM trial mouse pairs exhibit similar trends in weight progression

When drug and control groups were compared via mean weight increases over time, no significant trend variation was found at $p<.05$. While vehicle mice tended to maintain a slight advantage in weight as seen in Figure 8, this increase was insignificant, as growth still maintained the same trend as in drug mice.

Tenovin-6 4.5mM vehicle control mice exhibit insignificant higher grip scores than drug mice

At first glance at Figure 9, it was noticed that for the majority of measured weeks, vehicle control mice maintained higher grip test scores than drug mice. Drug mice did however maintain a consistent score trend whereas control mice exhibited a steady decline. Although the overall trends did not prove to be significantly different at $p<.05$, continued measurements until end stage phenotype coupled with more subjects could be more enlightening.

Figure 5

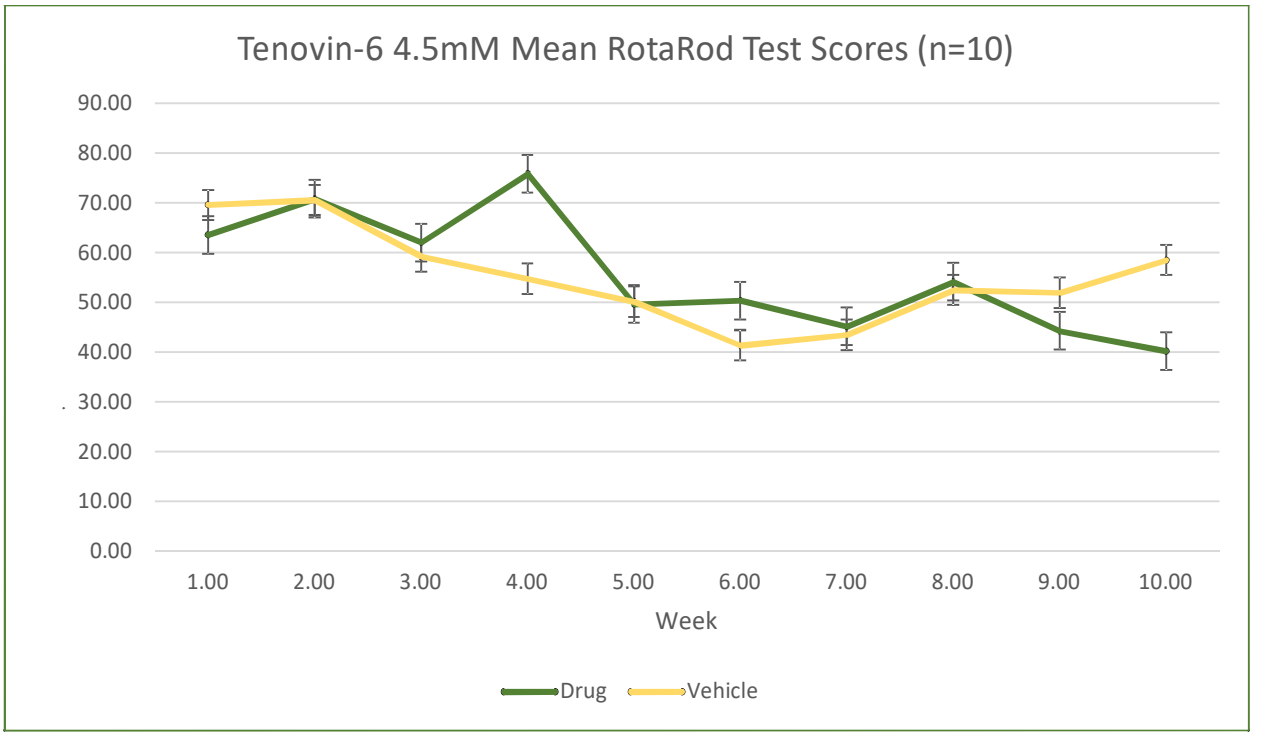

Figure 5: *Mean scores for both the drug and vehicle control groups were plotted on a line graph for trend comparison. When averaged out, both groups maintain similar trends of test scoring apart from week 4. At this 4.5mM tenovin-6 dosage, drug mice at week 4 displayed a marked improvement in rotarod scoring compared to their controls. This boost in score diminished the following week.*

Figure 6

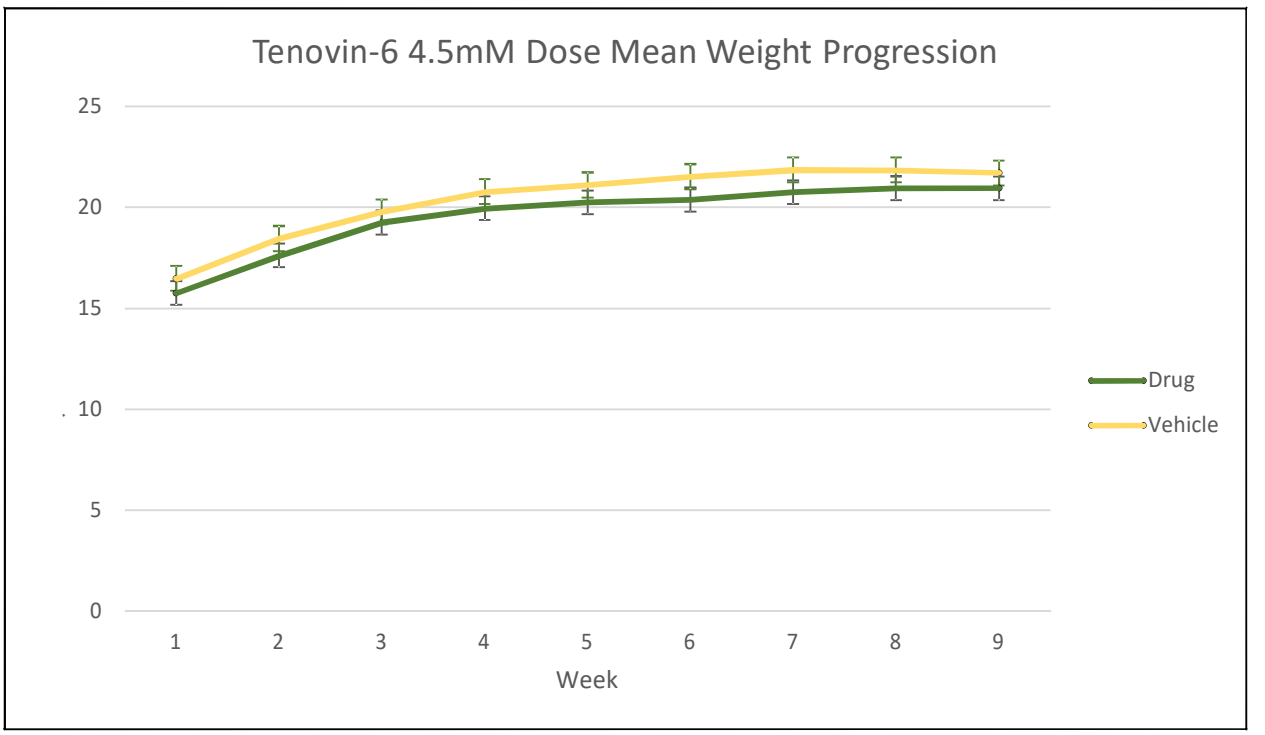

Figure 6: *Mean weights between trial pairs were plotted on a line graph for trend comparison. While the vehicle control group does maintain a higher weight throughout the experiment, the difference was found to be insignificant ($p<.05$). Additionally, both groups had nearly identical weight growth curves over time.*

Figure 7

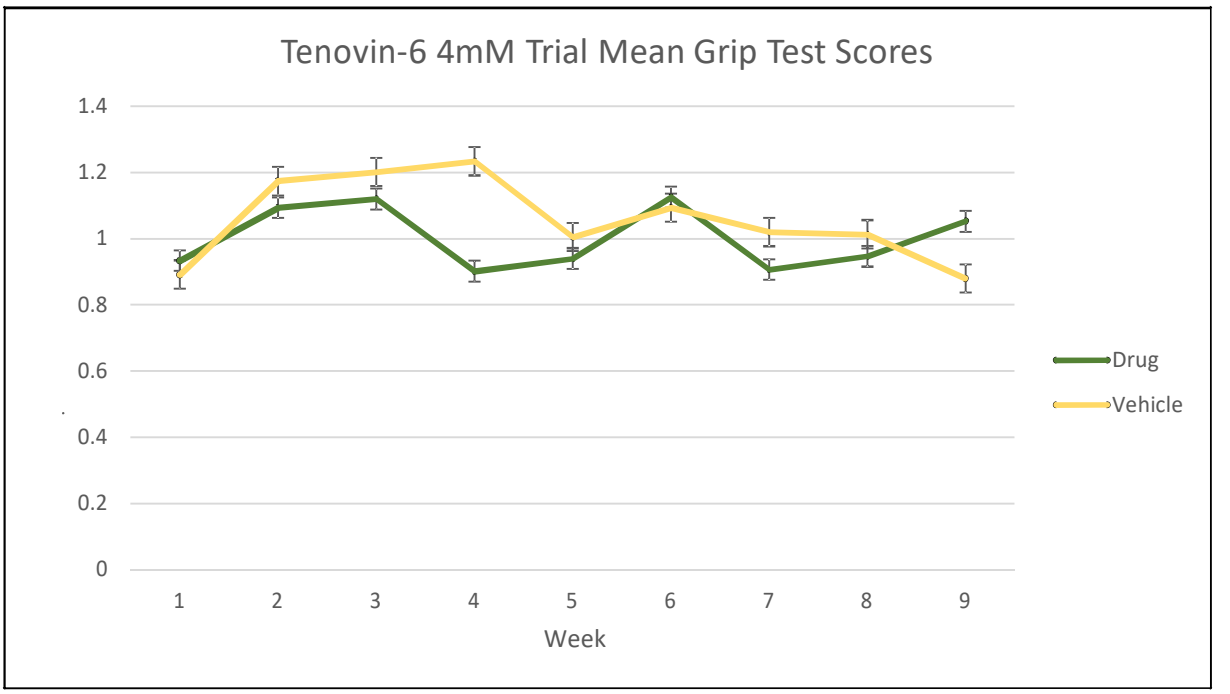

Figure 7: Mean grip test scores were taken and plotted on a line-graph to determine the presence of any trends over time. While score progression proved more erratic compared to RotaRod and weight score trends, it is worth noting the generally higher score of the control mice compared to the drug mice for the majority of measure weeks. Despite this, trending depicts drug mice maintaining a lesser but consistent score over 9 weeks whereas vehicle mice exhibit higher scores but a decrease in scores over a 9-week period. Nevertheless, at $p<.05$, no statistical difference was found.

Discussion

Given the effects of Tenovin-6 in vitro on p53 and protein quality control, the consequent goal was to observe if similar benefits would be seen in vivo. $SOD1^{G93A}$ transgenic mice, a common animal model used to study ALS in vivo seemed to be a good candidate for drug trial. Prior to formulation of each mouse trial group, genotyping was verified and all experimental and control pairs were both gender and litter matched. Tenovin-6 was the first drug to be tested with three separate low dose groups, as no clinical dose had yet been established for a mouse model. The water-insoluble variant Tenovin-1 had been previously used by another lab member in the year prior. However, due to unforeseen complications coupled with the significant insolubility of the drug, it seemed prudent to go ahead with a much soluble and effective variant. Despite the water-solubility of tenovin-6, initial drug trials adhered to a drug mix protocol devised by Ueno et al in 2014 to follow precedent. Having utilized tenovin-6 in vivo for cancer research, their mix called for drug diluted in PBS, 10% DMSO, and 20% cyclodextrin. While this protocol was adhered to for the tenovin-6 low dose trials as well as the WT baseline trials, they were abandoned for a simpler approach in the high dose tenovin-6 trials.

Early fears of tenovin-6 toxicity were partially the cause for such low initial drug dosages, given that cancer research utilized the compound to dysregulate autophagy in cancer cells (MacCallum, Groves et al. 2013). Nevertheless, once safe levels were introduced in vitro and converted to proper doses in mice, any concerns were alleviated. The resulting tenovin-6 trial data as well did not indicate any abnormal effects stemming from confounding drug effects. In addition, the drug was proven in vitro to clear $SOD1^{G85R}$ aggregates in transfected HEK293T cells. ‘

The low dose tenovin-6 trial proved to be excellent in terms of stability and absence of any confounding unforeseen variables. To further reduce any concerns regarding injection or vehicle

compound affecting behavior or testing, both a WT baseline group and an untreated SOD positive female group were created. The WT groups satisfactorily indicated that vehicle injections did not affect testing performance or weight gain. The SOD positive female group served as a fundamental baseline from which the drug trial groups would expectantly deviate from. However, given the insignificant differences between drug and control mouse score and weight trends, it seemed we may have potentially erred too much on the side of caution. Consequently, it was decided to significantly increase the dosage of tenovin-6 from the micromolar scale (80μM, 26.4 μM, 8 μM) to a much higher millimolar scale (4.5mM).

Compared to the low dose drug tenovin-6 groups, the new groups were more simplified in terms of protocol and also yielded a greater yet still subtle variation in results. To reduce the amount of exogenous substances being introduced, cyclodextrin was removed from drug mixes citing the high water-solubility of both drugs. Additionally, injection needles were preloaded with drug mixes at the time of first dilution to minimize the amount of thawing and freezing.

Tenovin-6 high (4.5mM) groups unfortunately did not display significant differences in trends. However, testing scores and trends did render as more erratic when compared to those of the lower dosage tenovins-6 trials. Given these changes, it was decided to begin a new tenovin-6 trial with a 13.2mM injection dosage. This dosage when administered to a 20g mouse registers at 30mg/kg. Unfortunately, either due to drug toxicity or user error, all drug mice (n=2) died after their first injections. Tenovin-6 has been noted in several studies to reduce and dysregulate autophagy in cancers, thus hinting at potential toxicity at very high doses (MacCallum, Groves et al. 2013). Additionally, given the need for proper protein quality control in ALS, a dysfunction in autophagy could potentially assist in causing lethality. Such findings and mechanisms will have to be studied further in the future.

In conclusion, it seems that while tenovins-6 clears protein aggregates in vitro, only very high dosage injections of it in vivo may confer a phenotypic benefit in $SOD1^{G93A}$ mice. Furthermore, it seems that given the potential toxic and lethal effects of tenovins-6 at very high doses. Ages of phenotypic onset coupled with testing trend analysis must be carefully monitored for any significant differences. As seen in tenovin-6, while higher dose injections may potentially improve motor functionality in ALS mice, such high concentrations could have other unforeseen mechanistic effects beyond the initial p53 upregulation. Thus it will be critical in the future to harvest and stain tissue appropriately for the correct biomarkers such as autophagy marker 1A/1B-light chain 3 (LC3) coupled with in-depth testing trend analyses. LC3 levels would be tested due to potential effects of the Tenovins on autophagy. If autophagy were dysregulated due to drug administration, levels of LC3 would be altered indicating disturbed autophagy-related processes (Tanida, Ueno et al. 2008). P53 protein levels would obviously need to be tested as well post-trial via tissue harvesting and staining.

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Curriculum Vitae

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Education

New York University, New York, NY **September 2010 - May 2015**

College of Arts and Sciences

B.A., Psychology

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

Department of Biochemistry and Molecular Biology **September 2014 – August 2016**

Sc.M., Biochemistry and Molecular Biology

- Thesis #1 – The Role of Mutant SOD1 Aggregates in ALS
- Thesis #2 – PROTEIN QUALITY CONTROL IN ANIMAL MODELS OF ALS

Georgetown University School of Medicine

Specials Masters in Physiology Program **August 2016 - Present**

M.S., Physiology

Clinical Experience

Prince William Hospital, Manassas, VA **July 2008 – June 2010**

Emergency Room and Intensive Care Unit Volunteer

- ER/ICU room maintenance
- Assistance with patient care and interactions
- Surgical procedure and specialist observation

Park Avenue Lasek, New York, NY **August 2012 – January 2013**

Medical Technician Intern

- Patient intake and vision analysis (Snellen eye exam, Wave/Orb scans, etc.)

- Variety of administrative tasks (medical history acquisition, medical chart completion, insurance verification)
- Educated in various ocular treatment methods and diseases (pre-laser surgery workup, post-surgery regimen, ocular exam interpretation)

Research Experience

NYU Center for Neural Science, New York, NY **July 2013 – July 2014**

The Ledoux Lab – Research Assistant

Faculty Mentor – Joseph E. LeDoux, Ph.D

- Involved and proficient in all areas of research including: Design, behavior testing, surgery, viral injection, brain cannulation, perfusion, IP injection, immunofluorescence, and data analysis
- Fear (threat conditioning) testing utilizing rats, studying neural pathways with viral markers

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

The Wang Lab – Research Assistant/Sc.M Student **October 2014 – August 2016**

Faculty Mentor – Jiou Wang, Ph.D

- Work and analysis of wild-type and mutant Ubiquilin-2 protein (PCR, transformation, expression, staining, etc.)
- Comprehensive analysis of transgenic G93A-SOD1 Black-6 mice and numerous others
- Maintenance/Harvesting/Experimentation of C9orf72 knockout mice (both full and conditional in motor neurons)
- Conducting several drug experiments related to the modulation of p53 in mice expressing ALS phenotype

Service

- Member of the SCOPE art magazine at Johns Hopkins
- Countless work at Emergency Rooms and Medical Offices/Clinics

Relevant Skills

Service

- Member of the SCOPE art magazine at Johns Hopkins
- Countless work at Emergency Rooms and Medical Offices/Clinics
- Organizing various Lab events and procedures

Initiative

- Initially planned to pursue a Ph.D in Clinical Psychology
- Particular interest in the neuroscience field, particularly in ALS, and the calcium hypothesis of neurodegenerative diseases
- Strong interest in the field of abnormal psychology and cognitive dissonance related discomfort/fear drives in improper cognition and behavior
- Strongly interested in the neurological underpinnings of maladaptive behaviors, ranging from disrupted pain/pleasure pathways to underdeveloped neurotransmitter-secretion circuits
- Interest in hybrid psychotherapies for treating wide spectrum mood disorders coupled with appropriate pharmaceuticals and other new technologies

Computer Familiarity

- Familiar with various computer programs such as Microsoft Excel, Serial Cloner, and SPSS

Effective Communication

- Years of experience interacting with both patients and clients, able to create a comfortable atmosphere but also clearly relay necessary information and readily provide assistance

Administration

- Experienced in general tasks ranging from organization to setting appointments in a medical/research setting

Language

- Moderate fluency in Spanish
- Learning/Improving Portuguese (Brazilian), Italian, Korean, Russian, and German

Teamwork

- Friendly and very easy to communicate and get along with even in a work/clinical environment

- Co-Captain of New York University Gaming Team – One of the top few hundred players in the country, top 1,000 in the world

Interests

- High-level physical fitness and nutrition
- Psychological theories of behavior and interaction
- Advances in the field of neuroscience (physiology of emotion and memories)
- Increasingly multilingual
- Playing/practicing musical instruments (trumpet, piano)
- All forms of music, especially classical music utilizing the harpsichord (Particularly Bach)
- Reading (Philosophy, fiction, education, theory, medical/psychiatry/neuroscience journals)